[*Reprinted from the Journal of Physiology,*
Vol. XLIV. No. 1 *&* 2, *March* 29, 1912.]

ON CREATIN-DESTROYING BACILLI IN THE INTESTINE, AND THEIR ISOLATION.

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In view of the fact that much of our knowledge of metabolism problems depends on feeding experiments, it is noteworthy that, in the interpretation of results, the possibility of the intestinal bacteria acting on ingested substances is seldom brought into consideration. It is obvious however, that this factor must be of importance, especially when it is remembered that micro-organisms are not confined to the large intestine but can be found in all parts of the alimentary tract, from the stomach downwards. Such a consideration is most urgent in the case of creatin and creatinin, because most of the evidence as to the part these substances play in the animal economy depends on experiments with the intact animal, and more especially on feeding experiments. But little information has been obtained by studying creatin metabolism with isolated organs, and this fact, taken in conjunction with the late appearance in nature of creatin and creatinin, namely with the vertebrates, suggests that their function is a highly specialised one, depending on the normal performance of many other chemical processes in the body. Apart from a general consideration, some results obtained by Folin[1], by feeding human beings with creatin under various conditions of dietary, raise the question as to whether bacterial action in the intestine is not an important factor in such experiments.

Folin's results may be briefly stated thus:—(1) creatin taken by the mouth, together with a diet containing small quantities of nitrogen,

[1] Folin. *Lancet*, Sept. 1906, p. 738.

is almost entirely retained in the body. Neither the creatin nor the nitrogen is excreted. (2) Creatin taken by the mouth, together with large quantities of nitrogen in the diet, is largely excreted unchanged.

Having noticed how susceptible creatin is to bacterial decomposition, one of us (E. M.)[1] suggested the possibility that Folin's results were due to the action of intestinal bacteria. It seemed probable that, when the diet contained but little nitrogen, the intestinal flora lived at the expense of the creatin nitrogen, with the result that neither the creatin nor its nitrogen was excreted; but on the other hand, when there was abundant nitrogen in the diet, the creatin was untouched by the bacteria because more assimilable substances were present, and the creatin on being absorbed from the intestine into the blood stream was ultimately excreted by the kidneys. With the object of demonstrating this possibility the following investigations were undertaken.

Preliminary experiments.

(*a*) In an earlier paper it was noticed that, when an extract of meat was exposed to the air, all the creatin disappeared in the course of a few days, and we thought that an examination of the bacteria present in such a medium might give an indication of the type of micro-organism responsible for creatin destruction in the alimentary canal. Films were made from a meat extract medium in which all the creatin had disappeared, and on staining, various cocci, Gram negative bacilli, and a few large bacilli were found. By means of ærobic plate cultivation at 37° C. pure growths of a number of micro-organisms were obtained, including micrococci, bacilli of the colon type, and some large Gram positive bacilli belonging to the Mesentericus group. When these bacilli were inoculated into a medium containing creatin (·5 % lemco, ·5 % sodium chloride, ·1 % sodium bicarbonate, ·1 % glucose, ·1 % creatin dissolved in distilled water) the creatin remained untouched. All attempts to isolate creatin destroying micro-organisms by this method were unsuccessful.

(*b*) In the next series of experiments we inoculated media containing creatin, with pure growths of certain well-known intestinal bacilli, and with other micro-organisms isolated from cases of colitis and cystitis. Two kinds of media were used: (1) The creatin medium described above. (2) A creatin-meat medium which consisted of the creatin medium with the addition of one gramme of muscle to 10 c.c.

[1] Mellanby. *Journ. of Physiol.* xxxvi. p. 471. 1908.

of medium. Each medium was distributed in ordinary sterile test tubes in quantities of 10 c.c. and sterilised in the usual way by discontinuous steaming. Tubes of each medium were inoculated with such bacilli as:—*Bacillus capsulatus* Pfeiffer, *B. cloacæ* Jordan, *B. pneumoniæ* Friedländer, *B. coli* Escherich, *B. lactis ærogenes* Escherich.

We also tested several Gram negative motile bacilli which fermented glucose and saccharose, and liquefied gelatine, but did not ferment lactose or dulcite. When allowed to grow for one or two days at 37° C. *Bacillus capsulatus* and *B. lactis ærogenes* were found to destroy part of the creatin—about five out of ten milligrammes were usually broken down. All the remaining bacilli gave negative results. From these experiments it was clear, that none of the bacilli tested could be regarded as of much importance from the point of view under consideration.

Examination of fæces for destructive micro-organisms.

First method. Using the creatin and creatin-meat medium described above, we inoculated tubes with minute quantities of fæcal material and placed them under ærobic and anærobic conditions at 37° C. We found that the creatin of the creatin-meat medium was completely destroyed in all the tubes. In the absence of meat however, the creatin was either left intact or only slightly broken down. Those tubes in which the creatin was destroyed were sub-cultured on to fresh tubes of creatin-meat medium, and here again the creatin was always completely destroyed. We then endeavoured to isolate the effective micro-organism from a tube in which the creatin had been destroyed, by making a number of plate cultivations on ordinary agar, glycerine agar and agar containing minced meat. Numerous colonies were isolated from the plate cultivations including varieties of the colon sub-groups, various micrococci, streptococci, diphtheroid bacilli, streptothrices, blastomyces, sarcinæ and moulds. These pure cultures were inoculated into the creatin and creatin-meat media, but all gave negative results with the exception of one variety of colon bacillus. This colon bacillus did not completely destroy the creatin in the medium, we found that 25–50 % always remained unchanged however long the period of incubation. Its action on creatin was most marked in the absence of meat. The bacillus proved to be Gram negative, and fermented glucose, lactose, dulcite and saccharose. We attempted to

increase its property of destroying creatin by growing it in the creatin medium with the various other micro-organisms isolated, and also by repeatedly sub-culturing on creatin medium, but without success.

From the above experiments, and especially from the observation that fæcal inoculation caused a rapid and complete disappearance of creatin only in the creatin-meat medium, whereas the creatin-destroying colon bacilli so far tested caused a partial disappearance of creatin only when meat was absent, we concluded that the micro-organism in the fæces responsible for this chemical change was not among those so far tested. Further, in view of the different conditions under which the fæcal micro-organisms and the isolated bacilli were found to act, it seemed probable that they did not even belong to the same group.

Second method. In the second and successful method for isolating the creatin-destroying micro-organism from fæces we proceeded as follows:—A medium was used which, while it allowed micro-organisms that destroy creatin to grow, was of such a nature that it was more difficult for those not possessing this special aptitude to survive. On repeated sub-culturing on to fresh tubes of identical medium the creatin-destroying micro-organism was relatively increased, with a corresponding diminution of other types.

It had been noted in earlier experiments, that in the creatin-meat medium in which the creatin was broken down a considerable number of large Gram positive bacilli were present, whereas in the creatin medium where the creatin was untouched, this particular bacillus was only present in comparatively small numbers, and tended to diminish in the sub-cultures. It seemed possible that this large Gram positive bacillus might be in some way responsible for the destruction of creatin, notwithstanding the negative results obtained with a large Gram positive bacillus isolated in the earlier experiments. It was also observed that the bacillus grew most abundantly at the bottom of the tube and particularly in the meat. It was reasonable to suppose therefore that this bacillus might be an anærobe, in spite of the fact that the creatin of the creatin-meat medium was always destroyed both under anærobic and ærobic conditions. In the ærobic tubes the abundant growth of the various types of colon bacilli also present probably sufficed to produce a condition of anærobis. It was found impossible to isolate the Gram positive bacillus by plate cultivation unless certain precautions were taken to prevent the motile colon bacilli from spreading over the plates. It was also found convenient to diminish the large preponderance of the colon bacilli, and to attain this end, meat extract and peptone were omitted

from the fluid medium, which now consisted only of a small portion of muscle in tap water. Tubes of this medium were sterilised and after boiling and cooling to 37° C., were inoculated with small portions of infected meat from the original tubes of creatin-meat medium in which all the creatin had been destroyed. The tubes were capped with gutta percha tissue and incubated for 2–3 days at 37° C. The fluid portion of the medium was then poured off and a portion of the muscle thoroughly washed with several changes of sterile normal saline. Sub-cultures were made from the centre of the piece of muscle on to a fresh tube of meat-water medium as before. The sub-cultures were made for four generations, and by this means the number of large Gram positive bacilli was considerably increased. From the last sub-culture a portion of muscle was removed and repeatedly washed with sterile salt solution. From the centre of this, plate cultures were made on to a medium consisting of ordinary peptone bouillon containing 2% agar and 2% gelatine. The gelatine was added to prevent the colonies of motile colon bacilli from spreading over the surface of the medium. The plates were placed in a Bulloch's anærobic apparatus, and in order to prevent the formation of condensation water the caustic potash and pyrogallic acid were placed in the dish dry. Hydrogen was passed through the apparatus, the internal pressure reduced with an air pump, and sufficient water run in to dissolve only a part of the pyrogallic acid. The cultures were incubated as usual at 37° C., and after a lapse of 24–48 hours were found to contain a fair number of discrete colonies, including a few which proved to be pure growths of the large Gram positive bacillus. From these colonies pure cultures were obtained on agar and glucose agar. The bacillus proved to be an anærobe and when inoculated into creatin media quickly destroyed all the creatin.

If other micro-organisms such as *Bacillus typhosus*, *B. fæcalis alkaligenes*, *B. coli* and *B. pneumoniæ* were inoculated with the anærobe, the creatin was effectively destroyed, even when the cultures were grown ærobically providing the tubes were capped with gutta percha tissue. The growth of the ærobic micro-organisms proved to be sufficient to remove any oxygen present in the medium.

This creatin-destroying bacillus also shows the following characters: in size and shape it is similar to the *B. ærogenes capsulatus*, motility is absent, and no spore formation has been detected. It is a strict anærobe, stains with ordinary aniline dyes, and retains the stain in Gram's method. It grows only slightly at room temperature, but very well at 37° C. and up to 50° C. or a little higher. The bacillus ferments

glucose, lactose and saccharose, but with dulcite only an occasional bubble of gas is formed. It grows best in the presence of solid muscle and glucose, but if the glucose be increased to 2% the micro-organism produces so much acid that it is soon killed. The cultures are best kept alive in ordinary peptone bouillon agar stabs maintained under anærobic conditions. The bacillus shows a close morphological resemblance to the *B. œrogenes capsulatus* of Welch, but a pure culture of this bacillus obtained from Král of Vienna was not found to affect creatin in any way.

The distribution of creatin-destroying bacilli in the intestine.

A cat was kept on a milk and bread diet for a month, and during the same period another cat was given meat. At the end of this time both cats were killed and the intestine exposed. The surface of the gut was sterilised with a hot spatula at different points from the duodenum to the rectum, and a small quantity of the intestinal contents removed with a Pasteur pipette and inoculated into tubes of creatin-meat medium. These tubes were placed at 37° C. and after four days incubation the creatin was completely destroyed in all the tubes. It may be noted that the milk diet had not eliminated the creatin-destroying micro-organisms even from the upper part of the intestine. In films taken from these cultures the large Gram positive bacillus was more abundant in the case of the meat-fed cat, but it could be detected in some of the cultures taken from the milk-fed cat.

These results, taken in conjunction with the isolation of specific creatin-destroying bacilli from human fæces, suggest that the human alimentary canal is inhabited by such bacilli for a considerable part of its length. Although our investigations by no means prove that the results obtained by Folin are due primarily to the action of intestinal bacilli, yet it is clear that bacterial action cannot be excluded when interpreting the results obtained in feeding experiments, particularly when creatin is the substance investigated.

The action of anærobic bacilli on creatin.

As already stated, the *B. œrogenes capsulatus* causes no destruction of creatin. Negative results were also obtained with:—*B. œdematis maligni, B. butyricus cadaveris, B. enteritidis sporogenes, B. anthracis symptomatici, B. botulinus.* Strangely enough the only well-known anærobic bacillus which was found to break down creatin was the bacillus tetani, which did so completely.

Summary.

(1) A method is described whereby a bacillus can be isolated from human fæces which can effectively destroy creatin. It is a large Gram positive bacillus and a strict anærobe. It acts best in a medium containing solid muscle. Bacilli of the colon group usually increase the rapidity of its action, probably by producing more perfect anærobis.

(2) A few varieties of bacilli, all lactose fermenters, belonging to the typhoid-coli group (colon group) can also destroy creatin, but the destruction is never complete.

(3) In the intestinal contents of a cat, fed on either a milk or a meat diet, are found micro-organisms which destroy creatin. These are not confined to the large intestine but inhabit the small gut up to the duodeno-jejunal flexure.

(4) The results obtained render it necessary that the action of intestinal bacteria must be considered in the interpretation of creatin feeding experiments.

(5) The tetanus bacillus, one of many other anærobic bacilli tested, destroys creatin.

We are indebted to the Royal Society for the Government Grants which partly paid the expenses of this research.